1 Hematite photoanode co-functionalized with self-assembling melanin 2 and C-phycocyanin for solar water splitting at neutral pH

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Abstract

Nature provides functional units which can be integrated in inorganic solar cell materials, such as light harvesting antenna proteins and photosynthetic molecular machineries, and thus help in advancing artificial photosynthesis. Their integration needs to address mechanical adhesion, light capture, charge transfer and corrosion resistance. We showed recently how enzymatic polymerization of melanin can immobilize the cyanobacterial light harvesting protein C-phyocyanin on the surface of hematite, a prospective metal oxide photoanode for solar hydrogen production by water splitting in photoelectrochemical cells. After the optimization of the functionalization procedure, in this work we show increased reproducible hydrogen production, measured parallel to the photocurrent on this bio-hybrid electrode in benign neutral pH phosphate. Over 90% increase compared to the photocurrent of the pristine hematite could be achieved. The hydrogen evolution was monitored during the photoelectrochemical measurement in an improved photoelectrochemical cell. The C-phyocyanin-melanin coating on the hematite was shown to exhibit a comb-like fractal pattern. Raman spectroscopy supported the presence of the protein on the hematite anode surface. The stability of the protein coating is demonstrated during the 2 h GC measurement and in an *operando* 24 h current density measurement.

Keywords

Solar water splitting, artificial photosynthesis, C-phyocyanin, hematite, melanin, pattern formation

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83 **Introduction**

84 The use of solar energy to satisfy the increasing energy demand has been pointed out by Ciampi
85 more than 100 years ago [14]. About 4 decades after the pioneering photoelectrochemical wa-
86 ter splitting on titanium oxide by Honda and Fujishima (1972), photoelectrochemistry for solar
87 hydrogen production in photoelectrochemical (PEC) cells is a resurging field [24, 45, 46]. Hema-
88 tite (α -Fe₂O₃) satisfies many requirements for a good photocatalyst and is thus a prospective pho-
89 toanode material for PEC water splitting [6, 32]. Interestingly, the actual conversion efficiencies
90 for hematite are lower than predicted by theory. The conduction band of hematite is below the
91 electrochemical water reduction potential and therefore needs a small bias voltage in a PEC cell. A
92 high rate of electron-hole recombination due to its short hole diffusion length is believed to be the
93 most probable cause for the low efficiency [38]. Most of the solar photons between 450 – 570 nm
94 are absorbed in hematite via an indirect band transition. These photons have deep penetration and
95 therefore can generate holes beyond the depletion layer; hence they recombine fast with the elec-
96 trons before they arrive to the surface. The performance of pristine hematite can be improved by
97 increasing the semiconductor - electrolyte interface area via nanostructuring [7, 39, 46], cation
98 doping [43], or by surface functionalization with proteins [8, 9, 21, 28, 39, 40] which can enhance
99 electron injection into the conduction band.

100 Artificial photosynthesis mimics natural photosynthesis, a process that converts sunlight, water,
101 and carbon dioxide into oxygen and carbohydrates. Various approaches [1, 3, 8, 30, 34, 39, 40]
102 have been made to combine semiconductor electrodes with natural light harvesting structures to

mimic photosynthesis. The aim is to build a device for converting the energy from sunlight into the chemical bonds of a fuel. In the prokaryotic cyanobacteria and eukaryotic red algae, light harvesting is performed by phycobilisomes [26]. Phycobilisome is a light harvesting complex, consisting of a supramolecular complex of phycobiliproteins, which are macromolecular protein complexes like phycoerythrin (PE), phycocyanin (PC) and allophycocyanin (APC), having different chromophores [10, 33]. The chromophore in PC is a linear tetrapyrrole molecule called phycocyanobilin (PCB), absorbing between 590 - 640 nm, when assembled in proteins [5]. An absorbed photon can initiate electron excitation and transfer. The energy is funneled to the reaction center and converted into chemical energy in photosystem II [17, 25, 47].

We have recently optimized a method using enzymatic polymerization of melanin to functionalize the hematite photoanode with a stable protein layer [21]. Tyrosinase oxidizes L-tyrosine and other mono-phenols to a reactive quinone species at the expense of molecular oxygen [22]. In the case of L-tyrosine the reaction product L-DOPA quinone spontaneously polymerizes and forms melanin [23]. This black pigment is a light-excitable organic semiconductor [11] that absorbs over the entire visible solar spectrum. The basic idea of using tyrosinase-catalyzed melanin formation in presence of PC, is to integrate PC in the polymer structure of melanin, to have a mechanically and biologically stable protein coating on the hematite. Beside the light harvesting properties of PC, the water splitting performance of the bio-functionalized hematite could be maintained or even be enhanced due to the semiconducting properties of melanin itself [11].

Energy generating hybrid systems were already described in the literature. The Bacteriorhodopsin/TiO₂ nanotube arrays hybrid system shows 50% enhancement in the photocurrent density compared to the bare TiO₂ [1]. A Chlorosome assisted TiO₂ columnar film-based biomimetic light harvesting device has also been developed. 30 fold enhancement in the photocurrent density was observed in the wavelength region from 640 nm to the near infra-red [37], however the reported photocurrent does not reach 40 μ A.

The hybrid system discussed in the present work shows substantially better photoelectrochemical performance than the pristine hematite film and additionally does not depend on the extra Nernst potential from a strongly alkaline electrolyte. This novel method allows for operation of the PEC photoanode at neutral pH while at the same time increasing the photocurrent to even higher values than with direct non-enzymatic C-phycocyanine functionalization [8]. The improved PEC cell allowed stable and reproducible photoelectrochemical hydrogen generation.

2 Materials and methods

2.1 Materials

Iron nitrate nonahydrate ($\geq 98\%$), tetrahydrofuran (THF, $\geq 99.9\%$), 1,4-dioxane (99.8%), 1,1'-carbonyldiimidazol (CDI, $\geq 97\%$), NaCl (5 mol L^{-1}), H_2KPO_4 (1 mol L^{-1}), HK_2PO_4 (1 mol L^{-1}), KOH (95%), 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, $\geq 98\%$) and C-phycocyanin (PC, 99.9%) isolated from *Spirulina* sp. were purchased from Sigma-Aldrich and were used without further purification. Oleic acid ($\geq 60\%$) and L(+)-tartaric acid ($\geq 99.5\%$) were Fluka products. Tyrosinase was produced with recombinant *E.coli*. [22, 41].

2.2 Precursor synthesis and hematite film deposition

Hematite films were prepared as described previously [7]. FTO glass slides (12 x 30 x 2 mm, TEC-8 from Hartford Glass Inc.) were dip coated in the precursor solution (DipMasterTM-50, Chemat Technology Inc., USA). The precursor solution was removed from the back of the glass with THF-soaked wipe and dried at 75°C for 5 min on a hot plate. The samples were then further annealed for 30 min at 500°C. Dip coating and annealing were repeated three more times to obtain four layers of hematite with $510 \pm 65\text{ nm}$ film thickness.

2.3 Protein immobilization

For the covalent attachment of proteins to the photoanode, the pristine hematite film was first conditioned with agarose and then activated with CDI, as described previously [8]. This conjugate [4] can either react directly with the primary amine groups of PC, or indirectly, by the enzymatic polymerization of L-tyrosine with tyrosinase, subsequently forming melanin. These activated electrodes were then treated in two different ways to obtain a melanin (A) and a melanin-PC (B) coated surface. We have used an optimized procedure described previously [21]. The reaction steps are illustrated in Fig. 1.

Figure 1. Scheme of the reaction steps for hematite photoanode functionalization.

A: Melanin synthesis with tyrosinase from L-tyrosine

0.1 mg mL⁻¹ L-tyrosine solution in PBS was prepared. 5 µL of the tyrosinase stock solution (10 mg mL⁻¹ in 10 mM Tris HCl pH 8.0) was added to 1 mL L-tyrosine. The activated hematite surface was covered with 200 µL of this mixture for 3 h. The electrodes were then rinsed with phosphate buffer saline (PBS) and with distilled water to remove any potentially unbound polymer and excess reactants.

B: Enzymatic cross linking of PC with tyrosinase in presence of L-tyrosine

1 mg PC was added to 5 mL 0.1 mg mL⁻¹ L-tyrosine PBS solution. 5 µL of the tyrosinase stock solution (10 mg mL⁻¹) was then added to 1 mL tyrosine-PC solution. The *activated* hematite surface was covered with 200 µL of this mixture and incubated for 3h at room temperature. The electrodes were then washed as described above.

2.4 Photoelectrochemical measurements

For the photoelectrochemical assessment of the photoanode, we followed the recommendations provided in [12], when not otherwise stated. Linear voltammetry was conducted in dark and under illumination using a specifically designed photoelectrochemical cell made of polyether ether ketone (PEEK) and Plexiglas (Fig. 2), and a potentiostat (*Voltalab80 PGZ 402*). The hematite photoanode was connected in a three electrode configuration as the working electrode. A platinum plate was set as counter electrode and an Ag/AgCl (with sat. KCl) electrode was used as a reference electrode. The electrodes were immersed in 1 mol L⁻¹ KOH (pH 14) or PBS (0.05 mol L⁻¹ H₂KPO₄/HK₂PO₄, 0.15 mol L⁻¹ NaCl, pH 7.2). Simulated sunlight was provided by *1 Sun Oriel Lamp* from *L.O.T. – Oriel AG*, corresponding to AM 1.5 global standard solar spectrum. The applied bias potential was 0.6 V and 1.1 V (vs. Ag/AgCl) for KOH and PBS electrolytes, respectively. The higher external bias in case of PBS was necessary because the lower pH of 0.05 mol L⁻¹ PBS provides less chemical bias than 1 mol L⁻¹ KOH [34, 42]. 2 h stability measurements (chronoamperometry) were performed in the above described Plexiglas PEC cell at 1.0 V (vs.

Ag/AgCl) in PBS, where the H₂ formation rate is the highest under the applied experimental conditions. At the same time the H₂ formation was monitored using gas chromatography (GC). Independently 24 h stability was measured using the PEEK PEC cell at potentials corresponding to the water splitting onset potential in KOH (0.2 V vs. Ag/AgCl) and PBS (0.6 V vs. Ag/AgCl). All results are the arithmetic average of at least 3 consecutive measurements.

2.5 Gas chromatography

The formation of H₂ was monitored with gas chromatography (GC) in the Plexiglas photoelectrochemical cell used for the electrochemical measurements. This allowed for parallel photoelectrochemical and GC measurements (Fig. 2). A GC-2014 instrument (Shimadzu) was used with a TCD detector and a Hayesep D 10' column. Temperatures of the injector, column and detector were 60, 40 and 140 °C, respectively. The generated H₂ was recirculated with Ar carrier gas using a pump at 0.1 bar overpressure, enabling the online monitoring of the H₂ evolution. 10 µL samples of the headspace were injected after selected reaction times. The flow rate on the column was 24 ml/min. The evolved H₂ was quantified based on calibration ($R^2 = 0.9997$) with 50, 100, 500 and 1000 ppm standard H₂ in Ar. The limit of detection (LOD) and the limit of quantification (LOQ) of the used GC method were determined to be 29.8 and 89.8 ppm H₂, respectively.

Figure 2. Left – PEC test station for solar H₂ generation and detection, with the PEC cell illuminated and in operation;
Right – PEC cell for in situ H₂ generation and online GC analysis.

2.6 Scanning Electron Microscopy

The morphology of the pristine and protein coated hematite films was investigated with a *Philips XL 30* scanning electron microscope (SEM) equipped with an energy-dispersive X-ray (EDX) detector (Oxford instruments). The accelerating voltage was 8-25 keV in high vacuum mode and a secondary electron detector was used. The quantitative protein and/or melanin coverage of the hematite photoanode surface was determined using the image processing software *ImageJ*.

2.7 Raman spectroscopy

The Raman spectra were recorded with a RENISHAW® in *Via Raman Microscope* using laser operating at 785 nm. The excitation light was focused on the sample through a 50X, 0.75NA objective. Raman scattering at 180° angle was collected through the same objective, then passed through a notch filter to reject the excitation wavelength and finally dispersed on a cooled CCD camera using a 1200 lines mm⁻¹ grating. For characterization of protein film distribution, Raman mapping of a 30 by 30 µm sample area was performed. Typical data acquisition time for one spectrum was 60 s.

3 Results and Discussion

Pristine hematite films are composed of nanoparticles with an average size of 50 nm and have a highly porous morphology, as shown in the scanning electron micrograph in Fig. 3a. X-ray diffraction on the pristine hematite film (Fig. 3b.) showed the typical Bragg reflections ((104) and (110)) for hematite.

Figure 3. a) SEM micrograph of a representative pristine hematite film surface; **b)** XRD pattern of a hematite thin film electrode. The 104 and 110 reflections (arrows) are the most characteristic for hematite.

The photocurrent densities of the hematite electrodes for every processing step in alkaline and neutral electrolytes are compared in Fig. 4. The photocurrent density of the pristine hematite was $257 \mu\text{A cm}^{-2}$ at 1,5 V (vs. RHE) in the standard 1 mol L^{-1} KOH electrolyte (Fig. 4, left panel), and $186 \mu\text{A cm}^{-2}$ at 1,6 V (vs. RHE) in 0.05 mol L^{-1} PBS (Fig. 4, right panel). The pH difference of ~ 7 of the two different electrolytes amounts to a Nernst potential [31, 34, 42] which causes a corresponding shift of the onset potential on the ordinate axes, when plotted against Ag/AgCl (sat. KCl) reference electrode (not shown here). The onset potential of the water splitting in PBS is shifted to $\sim 0,4 \text{ V}$, compared to that, measured in 1 mol L^{-1} KOH, due to less chemical bias in the former case. Coating the pristine hematite with melanin yields only an insignificant increase of the photocurrent from 257 to $276 \mu\text{A cm}^{-2}$ in KOH, whereas in PBS this increase is about 25%, to $236 \mu\text{A cm}^{-2}$. In alkaline electrolyte the photocurrent density of the hematite that was further functionalized with both, melanin and PC ($264 \mu\text{A cm}^{-2}$) does not differ significantly from the melanin-only coated and pristine hematite. However, in PBS, the melanin-PC functionalized hematite yields a photocurrent density of $360 \mu\text{A cm}^{-2}$. This is almost a twofold enhancement (93%) compared to

the pristine electrode in PBS, and still a 35% enhancement when compared to all the three electrodes measured in strongly alkaline standard electrolyte.

Fig. 4. Current density of the pristine hematite (—), melanin coated hematite (—) and melanin-PC coated hematite films (—) recorded in 1 mol L⁻¹ KOH (left panel, pH ~14.0) and 0.05 mol L⁻¹ PBS (right panel, pH ~7.2). The black curves show the dark current density.

The significant difference in the photocurrent density for the pristine hematite film and the one functionalized together with melanin and protein was confirmed by monitoring the H₂ evolution with GC (Fig. 5a).

The H₂ formation rates for pristine, melanin coated and PC-melanin coated hematite photoanodes (Fig 5, left panel) were calculated based on linear fit of the data measured after the first 20 minutes. During the initial 20 minutes the H₂ formation rate was low. We assume this time was needed for saturation of the electrolyte with the formed H₂. After reaching saturation the H₂ diffuses into the headspace of the PEC, from where it is flushed into the GC with Ar.

The H₂ formation rates for the pristine hematite, melanin coated hematite and PC-melanin coated hematite are 11.7, 11.2 and 18.3 ppm/min, respectively. The difference between the former two values is not significant; melanin itself does not contribute strongly to the H₂ evolution. Using the functionalized hematite electrode, after 2 h the H₂ concentration was 2200 (170) ppm of the. This is about 60% more than the 1400 (100) ppm formed using the pristine hematite photoanode.

Figure 5. Left panel: H₂ evolution on pristine hematite (▲), melanin coated hematite (■) and PC-melanin coated hematite (◆) photoanodes during a chronoamperometric measurement at 1.6 V (vs. RHE) in 0.05M PBS; right panel: chronoamperometry at 1.6 V (vs. RHE) in 0.05M PBS on pristine hematite (lower red line), and PC-melanin coated hematite (upper green line) photoanodes, measured while following the H₂ evolution by GC measurement.

Note that the photoanode in fact evolves O₂ from water splitting, whereas the H₂ is evolved at the counter electrode. Notwithstanding, the H₂ evolution at the Pt counter electrode is partly reflected by the photocurrent evolution over time at the photoanode, when we compare photocurrent and H₂ formation for the pristine and melanin-PC coated hematite. The difference observed between the photocurrent and H₂ concentration increase in PBS can be explained by the portion of H₂ staying dissolved in the electrolyte during the measurements. Chronoamperometric measurements show that the protein coating was stable during the ~2 h GC measurements (Fig. 5b). In an independent, 24 h measurement, a decrease of ~15 % in photocurrent density was measured, after the system reached the equilibrium.

We have recently shown, that the combination of adsorption and covalent immobilization of PC to hematite photoanode results in a two fold increase of the photocurrent density in 1 mol L⁻¹ KOH, compared to the photocurrent achieved using pristine hematite [8]. The chromophores remained active even in strong alkaline conditions (pH 14), when attached to a hematite support. This situation cannot be assessed in the present work because melanin dissolves in KOH [3], which would lead most probably to a disintegration of the protein network. Even if the chromophores remain in-

tact in the alkaline electrolyte, the dissolution of the melanin would interfere with the binding of the PC onto the hematite photoelectrode. Consequently, charge transfer between PC and hematite is not warranted anymore. This explains the insignificant (2-5 %) differences in the measured photocurrent using 1 mol L⁻¹ KOH (Fig. 4, left panel). However, in PBS as electrolyte, the melanin coating stays stable and even contributes slightly (25%) to the photocurrent (Fig. 4., right panel). An explanation for this observation could be the semiconducting property of melanin itself [11]. The enzymatic melanin-PC coating caused a significant, 93% enhancement in photocurrent density in neutral electrolyte. Beside this impressive increase of the photocurrent density, further advantage of this hybrid photoanode is that it works in pH neutral electrolyte. This shows that iron oxide based water splitting devices can be fabricated and used also in a protein friendly, and thus, generally more benign environment [35]. For the development of a solar energy conversion system in public areas and domestic building, environmentally benign components, i.e. PBS rather than concentrated KOH, are more likely to be accepted.

The electron micrographs in Fig. 6a and b provide a visual impression of the morphology of the protein coated (mel-PC) hematite films. The calculation of surface coverage showed ~20% for the melanin and ~50% for the PC-melanin coated hematite films. Most interestingly, the melanin-PC organic layer has arranged in a regular, self-similar pattern with a comb-like architecture on the hematite. The magnified image on the right (Fig. 6b) shows that the comb branches are aggregates from globular primary particles of around 200 nm in diameter. The aggregate branches range from 500 nm to 5 µm in length. The reason for the high structural organization could be the specific assembly of the PC-melanin polymer. Hematite films functionalized with only melanin are shown in Fig. 6c and d. Melanin organizes in a different pattern when PC is not present, forming more random bow-like branches, where the branches consist of nanoparticle chains similar in morphology described in an earlier study [16]. The magnification (Fig. 6d) shows that melanin constitutes primary particles as almost flat platelets with dumbbell shaped morphology of 3 to 5 µm average

size, aggregated to fractal-like branches of 10 to 50 μm , substantially different from the PC-melanin structure, both in size and in topology. The aggregation and fractal formation of melanin have been recognized before [20, 27, 29]. Whether melanin arranges in an ordered or amorphous structure depends on a delicate balance of the synthesis parameters. Davidenko *et al.* showed the formation of both, amorphous and ordered (dendritic) melanin films [13].

Fig. 6. SEM micrographs of PC-melanin (a and b) and melanin-only (c and d) coated hematite films surfaces with 2 different magnifications.

Highly organized structures are formed only in the presence of PC, and may potentially be responsible for the high increase of the photocurrent due to optimized orientation of the chromophores; [15, 18]. PC is known to form trimers, which are able to assemble into hexamers and further into

rods. This was shown to be functionally important, since it determines the distance and angles of each chromophore relative to its nearest neighbors, which defines the most probable pathways of energy transfer [36]. However the elucidation of this question is beyond the scope of the present study.

Raman micro-spectroscopy was performed in order to specify the distribution of the self-assembled protein structure on hematite. A 30 μm by 30 μm area was mapped with a resolution of 1 μm . The comb-like patterns known from the SEM micrographs in Fig. 6 are partially visible in Fig. 7a. The three upper Raman spectra shown on the right represent the overall Raman scattering of the material probed, which is reflected by color code in the Raman image. The most prominent spectral feature of the protein film corresponds to the amide II band centered around 1550 cm^{-1} . There is a weaker band at about 1388 cm^{-1} , which can be attributed to the amide III vibrational mode. The spectrum of the pure melanin [2] overlaps to some extent with the protein spectra [44]. A spectrum of pristine hematite film (Fig. 7c, bottom black curve) is used as a reference with the typical hematite peaks at 211, 294, 410, 496 and 611 cm^{-1} [8, 19]. The band at around 1327 cm^{-1} originates from the glass substrate. Most importantly, the protein attributed peaks in the 1400-1600 cm^{-1} region are clearly absent in the spectrum of hematite whereas the spectra of the hybrid samples contain the features of both hematite and protein. To create a Raman map, the intensity of the amide II band was plotted after fitting the spectra for each data point. By comparing panels (a) and (b) in Fig. 7, one can clearly observe the correlation between the self-assembled protein structure located in the center of the optical image and the Raman map. Notably, Raman analysis shows that the protein film in the hybrid structure is concentrated enough to exhibit measurable Raman scattering even in the areas, which appear to have very thin protein coating based on the optical images of the sample.

The slight intensity shoulder at 1610 cm^{-1} coincides with a prominent band recently observed in C-phycocyanin Raman spectrum [8]. Not shown here – subtraction of the hematite spectrum from samples with melanin and melanin-PC films shows also this feature for the PC coated sample. This shoulder is well noticeable in the first (red) spectrum from the top in Fig. 7, barely noticeable in the

spectrum below (green), and absent in the second spectrum from the bottom (blue) and in the pristine hematite spectrum. It is thus possible that the blue region in the Raman mapping contains melanin but no PC.

Figure 7. a, Optical image of the sample delineating the area in the center which was characterized by Raman spectroscopy. **b,** Raman map based on the intensity distribution of the 1550 cm^{-1} peak. Panel **c,** shows the representative Raman spectra corresponding to the highest (red line), intermediate (green line) and lowest (blue line) intensity of the protein bands in the 1400-1600 cm^{-1} range. The bottom graph (black line) shows the spectrum of pristine hematite on FTO glass.

However, the generally close proximity of the characteristic bands of melanin and PC does not allow a final conclusion about the relative spatial distribution of melanin and PC on such highly porous hematite substrate.

Conclusion

In this work we show an optimized enzymatic formation of melanin that can be utilized for immobilizing the cyanobacterial light harvesting protein PC on the surface of the hematite photoanode, enhancing the efficiency of the photoanode. Upon addition of tyrosinase to a PC-L-tyrosine mixture on hematite surfaces, a melanin-PC network is formed which is cross-linked to the hematite surface. This modification resulted in an enhancement in photoelectrochemical performance by a factor of two. The melanin-PC coating shows a fractal structure, which might contribute to the observed photoelectrochemical efficiency. At this point it remains open whether this fractal structure is functional or coincidental. Enhanced stable and reproducible H₂ formation rates were observed for an improved lab-scale PEC cell with melanin-PC coated hematite photoanode. A technological and societal benefit of the demonstrated system is its operability under benign conditions at neutral pH.

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Acknowledgement

The authors wish to express their deepest and sincerest recognition of Prof. András Dombi a key figure in the topic of photocatalytic materials for the degradation of contaminants of environmental concern.

Funding for this research was provided by the Swiss Federal Office of Energy project No. 100411, the Seventh Framework Program “Novel Materials for Energy Applications” grant No. 227179 (NanoPEC), the Swiss National Science Foundation R’Equip No. 206021-121306 and IZK0Z2-133944, the Swiss State Secretariat for Education and Research project Sciex 10.013 (NIPSH) and the VELUX Foundation (no° 790, BioPEC). This work was performed, in part, at the Center for Nanoscale Materials, a U.S. Department of Energy, Office of Science, under Contract No. DE-AC02-06CH11357. The authors acknowledge Dr. Tzu-Wen Huang and Dr. Songhak Yoon for the XRD measurements. The PEEK PEC cell was built by Peter Wyss and Marc Zollinger (Empa Machine Shop) after a design provided by Laboratory for Photonics and Interfaces, EPFL Lausanne. Heinz Altorfer assisted with the design of the Plexiglas PEC cell. Graphic enhancement was done by Philipp Rogenmoser, Empa Graphics Department.